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Isolation and Identification of Vesicular-Arbuscular Mycorrhiza-Stimulatory Compounds from Clover (*Trifolium repens*) Roots

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Two isoflavonoids isolated from clover roots grown under phosphate stress were characterized as formononetin (7-hydroxy,4′-methoxy isoflavone) and biochanin A (5,7-dihydroxy,4′-methoxy isoflavone). At 5 ppm, these compounds stimulated hyphal growth in vitro and root colonization of an undescribed vesicular-arbuscular mycorrhiza, a *Glomus* sp. (INVAM-112). The permethylated products of the two compounds were inactive. These findings suggest that the isoflavonoids studied may act as signal molecules in vesicular-arbuscular mycorrhiza symbiosis.

Vesicular-arbuscular mycorrhizae (VAM) result from a complex sequence of interactions between fungal hyphae and host cells, leading to a functional mutualistic state (6). Plant factors stimulate VAM hyphal growth in vitro and also the precolonization phase of VAM formation (4, 11, 13, 25, 26, 29). It has been suggested (14, 15) that the quantity rather than the presence of specific compounds in the root exudates is responsible for stimulation of fungal growth and VAM root colonization (36). Other studies, however, showed no relationship between root exudation and VAM infection (2). Viable spores of most VAM fungal species readily germinate on distilled water (39), and there is no evidence that they require any specific host factors. However, certain components of root exudates or plant cells may act as signal molecules capable of inducing hyphal growth, branching, differentiation, and host penetration (4, 5, 25).

An earlier report from this laboratory (11) indicated the presence of a transient VAM-stimulating factor in exudates from phosphorus-deprived young white clover seedlings. This study also indicated that the quality of the exudate is important in stimulating VAM hyphal growth. Similar results have been found with stressed suspension-cultured legume cells (29) and with cultures of Ri T-DNA-transformed roots (3–5). The studies with transformed roots also indicated that root-inducing factors are required for the fungus to switch from preinfection to a biotrophic state. However, the nature of these highly active plant factors has not been determined to date. In this study, we report the isolation and identification of VAM-stimulatory compounds from clover roots.

MATERIALS AND METHODS

General analytical methods. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 300 spectrometer in CD_3OD solutions at 25°C. Infrared spectra in KBr were obtained with a Perkin-Elmer 1600 series FT-IR spectrometer, and UV spectra were recorded on a Shimadzu UV-265 spectrometer. Melting points were recorded on a Thomas model 40 micro hot-stage apparatus and are uncorrected. Mass spectra (MS) were obtained on a JEOL model HX-110 mass spectrometer. Vacuum liquid chromatography and preparative thin-layer chromatography were carried out on silica gel adsorbants by using $CHCl_3$-methanol (MeOH) solvent systems.

Plant material. White clover (*Trifolium repens* L. cv. Ladino) plants (2 weeks old) were used for all exudate and extract collections. Plants were grown in sterile square glass staining dishes containing Hoagland solution with and without phosphorus as previously described (11). At 2 weeks of age, exudates were collected from the roots of clover seedlings and grown with or without phosphorus as follows. Plants were taken out of the nutrient solution and rinsed with sterile distilled water several times, and then 50 seedlings were placed in each staining jar containing sterile distilled water (50 ml) for 24 h. The distilled water in which seedling roots were immersed for 24 h was pooled for each treatment and lyophilized at 4°C. Contamination checks were done after 7 and 14 days during plant growth by plating out spent nutrient solution on water agar and potato dextrose agar at the time of nutrient solution replacement. No contamination was observed. The roots collected from the seedlings of the same experiment (14 days old), as well as root exudates, seeds, and plant tops, were lyophilized at 4°C and used for extraction.

Bioactive compounds from the lyophilized roots. Isolation and purification of the active compounds for VAM hyphal growth were carried out as shown in Fig. 1. The extract was purified by vacuum liquid chromatography as follows. Silica gel for column chromatography (40 mesh) (60 g) was made into a slurry in $CHCl_3$-MeOH (4:1, vol/vol) and poured into a sintered glass filter (fine) fitted with a Büchner flask connected to a vacuum line. When the slurry was almost dry under vacuum, a solution of the extract in $CHCl_3$-MeOH (4:1) was applied on the surface of the silica gel and eluted with the same solvent system (150 ml). The organic extract was dried in vacuo and further purified by preparative thin-layer chromatography. Two pure fractions thus obtained were used for bioassay and chemical characterization, and they were called clover A (R_f = 0.68, 3.6 mg), compound 1, and clover B (R_f = 0.60, 2.8 mg), compound 2. Identical fractions were obtained from the root exudates as well. Since the compounds were in larger quantities from the root extracts, further purification and identification of the active components were carried out only on root extracts.

Compound 1 (clover A). The high-R_f band, colorless needles from hot MeOH; mp, 255 to 256°C; UV (MeOH, nm)

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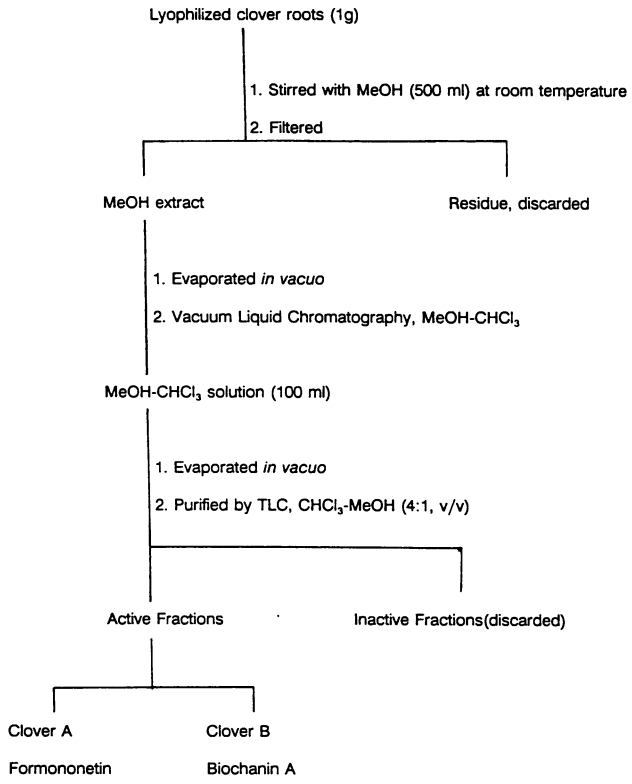


FIG. 1. Isolation and purification scheme for clover A (formononetin) and clover B (biochanin A) from 14-day-old white clover roots. TLC, Thin-layer chromatography.

212, 248, 300; +KOH 206, 256, 336; ^{1}H NMR (CD_3OD) δ 8.15 (1H, s, H-2), 8.05 (1H, d, J = 9 Hz, H-5), 7.45 (2H, dd, J = 2,9 Hz, H-2′, H-6′), 6.98 (2H, dd, J = 2,9 Hz, H-3′, H-5′), 6.85 (1H, dd, J = 2,8 Hz, H-6), 6.81 (1H, d, J = 2 Hz, H-8), 3.80 (3H, s, OMe); ^{13}C NMR (CD_3OD) δ 152.22 (C-2), 124.43 (C-3), 175.61 (C-4), 127.71 (C-5), 119.26 (C-6), 154.22 (C-7), 102.30 (C-8), 156.45 (C-8a), 122.16 (C-4a), 123.87 (C-1′), 130.01 (C-2′), 113.61 (C-3′). 159.20 (C-4′), 113.61 (C-5′), 130.01 (C-6′), 55.28 (OMe); Cl (+, methane)-MS, m/z (% int.) 269 (M+H, 100), 257 (12), 239 (5), 132 (8); El (+, Dl)-MS, m/z (% int.) 268 (M^+, 100), 253 ($M^+ - CH_3$, 40), 239 (20), 150 (35), 132 (100).

Compound 2 (clover B). The low-R_f band, pale-brown solid; mp, 212 to 214°C; UV (MeOH, nm) 210, 260; +KOH 271, 326; ^{1}H NMR (CD_3OD) δ 8.01 (1H, s, H-2), 7.40 (2H, d, J = 8 Hz, H-2′, H-6′), 6.90 (2H, d, J = 8 Hz, H-3′, H-5′), 6.35 (1H, d, J = 1.8 Hz, H-8), 6.25 (1H, d, J = 1.8 Hz, H-6), 3.75 (3H, s, OMe); ^{13}C NMR (CD_3OD) δ 155.78 (C-2), 117.20 (C-3), 178.30 (C-4), 160.30 (C-5), 116.92 (C-6), 160.80 (C-7), 103.50 (C-8), 160.10 (C-8a), 126.20 (C-4a), 55.80 (OMe); El (+, Dl)-MS, m/z (% int.) 284 (M^+, 100), 269 ($M^+ - CH_3$, 42), 204 (5), 180 (5), 156 (10), 132 (20).

Compound 3. Compound 1 (50 mg) was methylated (27) by dissolving in dry acetone (50 ml), stirred with K_2CO_3 (1.2 g), and refluxed (10 min). Dimethyl sulfate was added to this mixture (0.5 ml) and refluxed until no more starting material was shown by thin-layer chromatography (6 h). The reaction mixture was cooled, the solid was filtered off, and the filtrate was concentrated, diluted with water, and extracted with ethyl acetate (EtOAc). The EtOAc layer was washed with an $NaHCO_3$ solution and water and dried over anhydrous $MgSO_4$. Removal of the solvent afforded a brown solid which was recrystallized from MeOH to yield colorless platelike crystals, compound 3 (45 mg); mp, 155 to 156°C; El (+, Dl)-MS, m/z (% int.) 282 (M^+, 100), 267 ($M^+ - CH_3$, 35), 251 (3), 239 (15), 150 (35), 132 (100), 117 (20), 89 (20).

Compound 4. Compound 2 (50 mg) was methylated with dimethyl sulfate in acetone with K_2CO_3 as in the case of compound 1, and the work-up of the reaction mixture afforded pale yellow platelike crystals from MeOH, compound 4 (40 mg); mp, 158 to 160°C; El (+, Dl)-MS, m/z (% int.) 312 (M^+, 100), 295 (10), 283 (15), 266 (20), 180 (10), 156 (15), 137 (8), 132 (30), 117 (5), 89 (5).

Spore germination. An undescribed VAM, a *Glomus* species (INVAM-112) (24a), subsequently referred to as *Glomus* 112, was grown in sorghum (*Sorghum vulgare*) pot cultures in the greenhouse for 4 months and stored at 4°C before use. This VAM-infested soil was wet sieved (12), and spores were suspended by centrifugation in a Ficoll gradient (10, 35, 45, and 60%). Organic debris was carefully removed from the spore suspension by hand with a Pasteur pipette under a dissecting microscope. Fungal chlamydospores were then surface sterilized with a 1:1:1 solution of 2% (wt/vol) chloramine-T, 0.02% (wt/vol) streptomycin sulfate, and sodium lauryl sulfate. After incubation under vacuum for 30 min, chlamydospores were washed with sterile distilled water. Surface-sterilized chlamydospores were stored at 4°C until used.

A medium designed for root organ culture was modified to contain no phosphorus (11). Fractions obtained as in Fig. 1 were incorporated into the agar medium at 5 ppm. Only uncontaminated chlamydospores were used in our experiments. *Glomus* 112 spores (30 per plate) were transferred to five replicate plates for each root exudate fraction to be tested (11). All plates were incubated at 25 to 27°C in the dark. Hyphal elongation was monitored at 5-day intervals. Hyphal elongation data include the mean hyphal lengths per spore and consider only those spores that germinated. This experiment was repeated twice.

VAM colonization and plant growth. The effects of synthetic formononetin (prepared by American Cyanamid Co.) and biochanin A (Sigma Chemical, St. Louis, Mo.) on VAM formation and subsequent plant growth were examined in growth-chamber experiments. In all experiments, water-soaked clover seeds were pregerminated on filter paper, inoculated with *Rhizobium leguminosarum* biovar trifolii (peat-commercial inoculum—Nitragin), and transplanted at a rate of one plant per cell into plastic inserts with individual cells (4 by 5.5 cm), each containing 80 g of a sand-soil mix. The bulked sand-soil mix was steam sterilized for 2 h, air dried, and stored until use. Chemical analysis of the soil indicated a neutral to slightly alkaline pH, low to moderate fertility, and a low exchange capacity. The soil was inoculated with *Glomus fasciculatum* obtained from pot culture. Dried inoculum was incorporated thoroughly into the soil mix at rates sufficient to achieve a final spore density ranging from 2 to 4 spores per g of soil. Prior to transplanting, 10 ml of 5-ppm solutions of each test compound were delivered into each cell by a pipette dispenser, unless otherwise indicated. The isoflavonoid compounds were first dissolved in a small volume of methanol and then transferred to water. Solvent controls were also prepared by using similar amounts of methanol (0.02% of the final volume) in water. Plastic inserts were placed in plastic trays and transferred to a full-light growth chamber (400 μmol/m^2 per s) with a 14-h day and 30 and 25°C day and night temperatures, respectively. For each four-cell unit, a plastic petri dish bottom (90

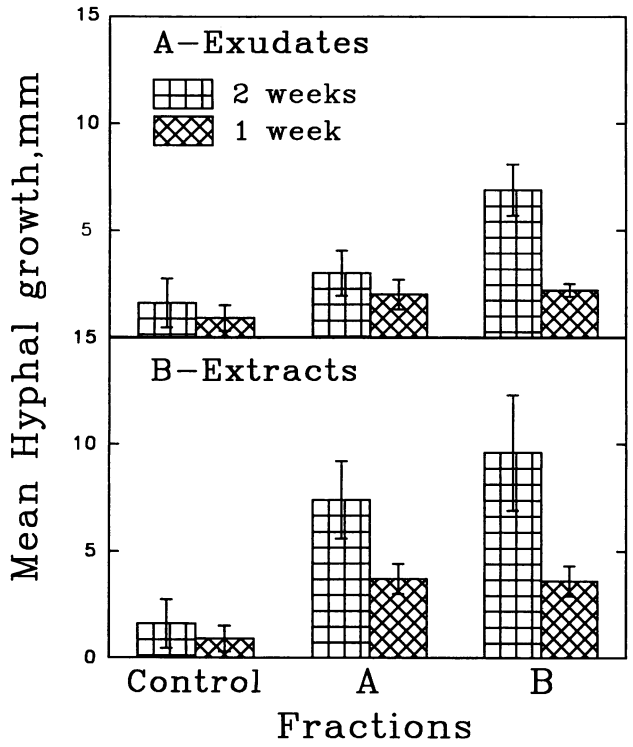
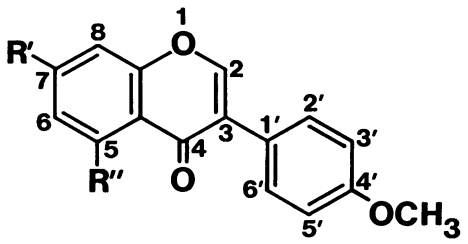


FIG. 2. Hyphal growth-stimulatory effects of clover A and clover B.

by 15 mm) was used as a base to avoid chemical contamination and to facilitate watering. Plants were watered daily with distilled water, from both the bottom and the top, and allowed to grow for up to 4 weeks. At the end of the growth period, plants were harvested and assessed for growth. Roots were washed free of soil and cleared, stained (31), and assessed for VAM colonization by a line intersect method (18). Experiments were conducted by using a completely randomized design with at least 12 plants per treatment and were repeated at least twice. Experimental data were subjected to statistical analysis by using an MSTAT statistical package (MSTAT-C, a microcomputer program for the design, management, and analysis of agronomic research experiments, 1988, Michigan State University, East Lansing, Mich.).

RESULTS

The purified fractions from lyophilized clover root exudates and extracts showed hyphal growth stimulation in vitro (Fig. 2). Growth stimulation was observed 1 week after spores were plated, and further increases were evident after 2 weeks. Root extract fractions were structurally identical to the exudate fractions, evidencing the presence of both formononetin (clover A) and biochanin A (clover B) in the exudates and in the extract as well (Fig. 3). An identical extraction procedure was carried out on clover tops and seeds used to produce roots for extraction. Analyses of the extracts from the seeds and plant tops did not indicate the presence of either clover A or clover B. These extracts were not active on VAM hyphal growth and hence were not investigated further. A similar lack of activity was found in extracts of roots obtained from clover plants grown in



1 Formononetin R′ = OH, R″ = H
2 Biochanin A R′ = R″ = OH
3 R′ = OMe, R″ = H
4 R′ = R″ = OMe

FIG. 3. Structures for clover A (formononetin), clover B (biochanin A), and their methylated products.

phosphate-supplemented Hoagland solution. It was interesting to observe that 90-day-old clover roots obtained under phosphate stress had lower concentrations of clover A and clover B (data not presented) and also showed the presence of another related isoflavonoid, which was not characterized because of its reduced activity on VAM hyphal elongation in vitro. The effects of synthetic isoflavonoids on VAM root colonization and growth of clover plants are given in Table 1. Both formononetin and biochanin A enhanced clover root colonization by *G. fasciculatum* and plant growth, whereas methylated compounds were inactive at the concentrations tested.

DISCUSSION

The stimulatory effects of growth roots on VAM are well documented (4, 5, 25, 26). In this study, for the first time we believe, compounds capable of stimulating hyphal growth and VAM colonization were isolated and identified. These compounds were identified as the isoflavonoids formononetin and biochanin A. Both are constitutively found in legumes, are derived from the phenylpropanoid pathway, and have also been regarded as phytoalexins (42).

Spectral analyses of both clover A and clover B confirmed the identity of these compounds as formononetin and biochanin A, respectively. ^{1}H NMR spectra of clover A and synthetic formononetin are identical (Fig. 4). The cross peaks observed in the long-range COSY experiment of formononetin in dimethyl sulfoxide-d_6 are assigned in Fig. 5. This experiment unambiguously proved that clover A is 7-hydroxy,4′-methoxy isoflavone, compound 1, and cannot

TABLE 1. Effects of synthetic isoflavonoids on growth and VAM root colonization of white clover plants after 4 weeks of growth in the presence of *Glomus* 112

Compounds	Clover top fresh wt (mg)[a]	VAM colonization (%)[a]
Control	38 c	33 bc
Formononetin	50 a	65 a
Biochanin A	49 ab	63 a
Compound 3	38 bc	36 b
Compound 4	34 c	23 c

[a] Means followed by the same letter did not differ by the least significant difference test at $P \leq 0.05$.

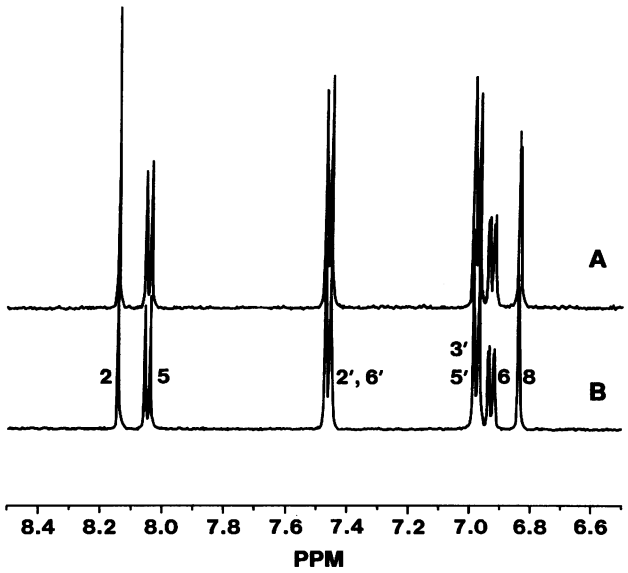


FIG. 4. ^{1}H NMR spectra (300 MHz) for clover A (A) and synthetic formononetin (B) in CD_3OD. The 4'-methoxy region in the spectrum is not shown.

be the isomer 7-methoxy,4'-hydroxy isoflavone. Long-range COSY experiments showed a strong, clear correlation of the methoxy group to the H-3' and H-5' protons and weaker correlation to the H-2' and H-6' protons (Fig. 5). The 7-OH group (δ 10.80) gave cross peaks with the H-5 proton as well. The 7-methoxy,4'-hydroxy isomer of formononetin had been synthesized earlier (21), and its spectral characteristics were different from that of clover A. Our ^{13}C NMR data on formononetin (clover A) and biochanin A (clover B) differ slightly from the published data (30) since we used only CD_3OD as the solvent instead of the CD_3OD–dimethyl sulfoxide-d_6 mixture. Clover B was identical to biochanin A in every respect. Methylation of both formononetin and biochanin A resulted in good yields of the permethylated products, i.e., compounds 3 and 4, respectively. It was important to prepare the methylated products of these compounds to ascertain the structural activity relationship to VAM stimulation. Synthetic formononetin and biochanin A, but not the methylated forms, caused a significant increase in VAM root colonization and growth of clover plants. The reasons why methylation eliminated the VAM-stimulatory effects of these isoflavonoids cannot be determined until the biological bases of the effects that these compounds have on VAM are elucidated.

Formononetin has been found as a stress metabolite in soybeans (28) and in the roots and forage of several legumes (8, 16, 18, 41). In forage, formononetin concentrations range from 14 ppm in alfalfa to 1,700 ppm in red clover (16). This compound was also found in greater quantities in clover root

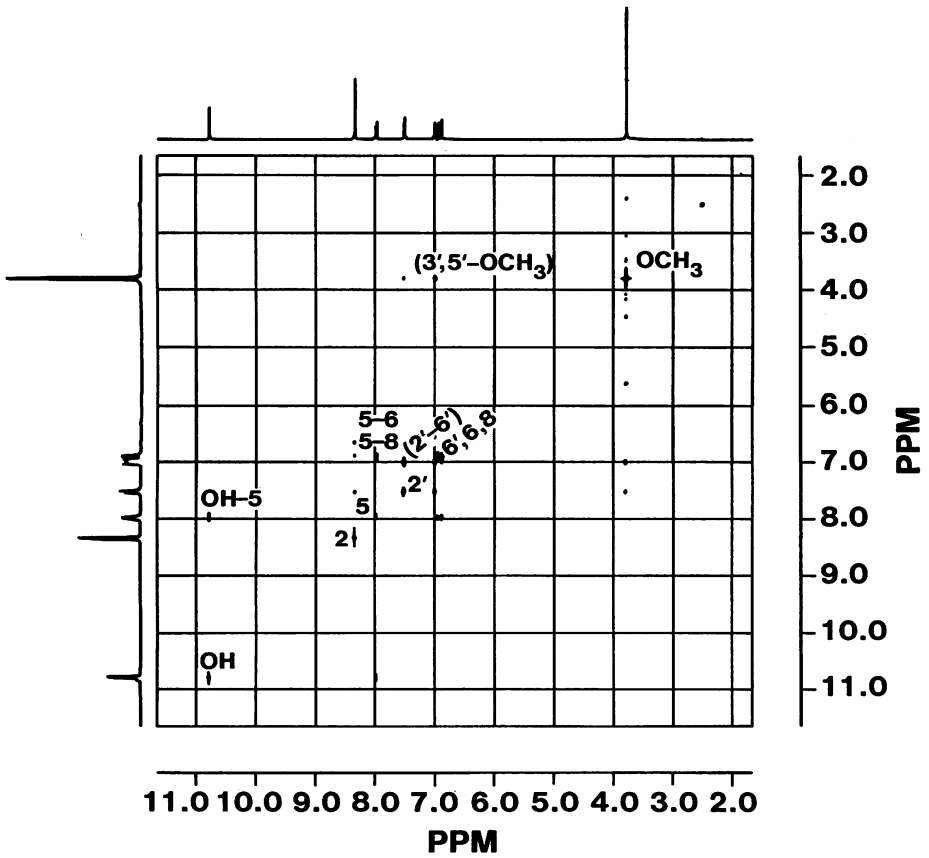


FIG. 5. Nuclear Overhauser effect spectrum for clover A obtained from a long-range COSY experiment in 100% dimethyl sulfoxide-d_6.

extracts than was any other *nod* gene-inducing flavone (10). Its concentration in clover plants is reduced by seedling age, light intensity, fertilization (34, 35), and plant pathogens.

VAM have been shown to increase accumulation of glyceollin and other isoflavonoids but not biochanin A and formononetin (23, 24). The activity of these two isoflavonoid compounds on fungal growth or VAM formation has never been studied, but they are known to be active on other fungi (43). Biochanin A has been shown to reduce growth of a *Rhizoctonia* sp. (44) and to stimulate growth of *Penicillium digitatium* (19). Formononetin effects on non-VAM fungi ranged from low antifungal activity to growth stimulation (9, 19). Other flavonoids have been shown to enhance growth of litter-decomposing fungi when applied at a concentration of 5 ppm (20). More recently, the flavonoids hesperetin and apigenin were shown to stimulate hyphal growth of the VAM fungus *Gigaspora margarita*, but their effect on root colonization was not reported (29). Hesperetin and other flavonoids are also considered "xenognosins" in root-infecting parasitic weeds (22); this association resembles a mycorrhizal association.

Since both formononetin and biochanin A were found in clover roots, increased hyphal growth in vitro by six- to ninefold over controls, and increased root colonization, these compounds may function as signal molecules in VAM symbiosis. The stimulatory effects of these isoflavonoids on VAM root colonization and plant growth have been confirmed in other studies with clover and other nonlegumes (37, 38) and are VAM mediated. When these compounds are applied in soil free of VAM propagules, they show no stimulatory effect on either plant growth or nodulation (37). In general, flavonoids function as signal molecules in *Rhizobium*-legume symbiosis by inducing transcription of bacterial nodulation genes (33). Nevertheless, neither formononetin nor biochanin A is a good inducer of *nod* gene activity on *Rhizobium leguminosarum* biovar trifolii. On the contrary, they may act as anti-inducers (10, 32). Since biochanin A and formononetin stimulate growth of non-nodulating plant species in the presence of VAM (37, 38), it is likely that they promote plant growth by stimulating VAM formation.

Mechanisms by which these isoflavonoids stimulate VAM formation cannot be determined from our work. Isoflavonoids have been suggested to alter membrane permeability, enzymatic activity, or DNA replication (1) in other fungi. In VAM, the isoflavonoids may act as (i) the plant signal that stimulates the free-living fungus to use its endogenous spore reserves (4), (ii) inducers for genes controlling symbiosis and saprophytic growth repressed as the result of coevolution (7, 40), and (iii) a plant signal for hyphal differentiation into appressorium and/or arbuscule (17) as in the case of parasitic weeds (22). Nevertheless, the increase in root colonization may simply result from the enhanced hyphal growth which may increase the fungus-root contact that is essential during the preinfection phase of this symbiosis.

These results along with those presented elsewhere (37, 38) constitute evidence that the isoflavonoids formononetin and biochanin A are involved in the stimulatory effects of clover roots towards the VAM. However, the definitive proof for the involvement of these compounds as signal molecules in VAM symbiosis requires further biological investigations.

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